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Characterization and Biological Activity of Essential Oils of Vaaltee, *Plecostachys serpyllifolia* Leaves

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ABSTRACT

Plecostachys serpyllifolia (Berg.) Hilliard is commonly termed Hottentot tea, trailing licorice or *Vaaltea*. It has been used as a remedy for cold and pains associated with the chest. The yield of essential oil extracted from 500 g of leaves of the plant by steam distillation was approximately 1.3% (v/w). The oil was analysed by GC-MS analysis. According to the analysis β-phellandrene was the dominant component followed by α-thujone. Extracts were positive against most gram positive bacteria tested. Extracts showed a range of inhibitory activity against fungal species tested. The methanol extract provided 100% inhibition against *Thamnidium elegans*, whereas the acetone extract showed 100% inhibition on *Rhizopus stolonifer*. This ability of inhibition of both bacterial and fungal species concurs with the broad spectrum antimicrobial activity of this plant species and could be attributed as to why *P. serpyllifolia* is often used in treatment of various diseases as a herbal or traditional remedy.

Keywords: antibacterial, antifungal, chemical components, β-phellandrene, α-thujone, GC-MS analysis
Abbreviations: alkaloid, flavonoid, flower colours, genetic transformation, metabolic engineering, secondary metabolism

INTRODUCTION

Plant species have been utilized as a source of food and medicine for millennia throughout the world and people have, from generation to generation, long identified themselves with vegetation types that are favoured by the geological and climatic conditions of their natural habitats (Shale *et al*. 1999; Koschier and Sedy 2003). Some of these plant species have been utilized as source of medicine as some of them have shown good antifungal, antioxidant and antibacterial properties under experimental conditions. Therefore, pharmaceutical companies and related industries are currently directing their businesses towards the use of plants, plant extracts or plant-derived phytochemicals to treat diseases or for use in the nutrition, perfume and aromatherapeutic industries (Kleining 1989; Auge *et al*. 2003; Nguefack *et al*. 2004). Most of these plants are well known for their nutritional, essential oil, fragrance and medicinal value.

Plecostachys serpyllifolia has been erroneously known as *Helichrysum microphyllum* but is synonymous with *Helichrysum orbiculare* or *Gnaphalium serpyllifolium* (Kelmanson *et al.* 2000). It is commonly known as trailing licorice, or *Vaaltee* (Afrikaans). The English common name is Hottentot tea and the plant grows in damp, sandy places, often near watercourses on the Eastern Cape region of the Cape province Cape Mountains. It also grows on the lower slopes of the mountains. *P. serpyllifolia* is a much branched straggling sub-shrub with slender branches. Its stems and leaves are covered by densely arranged whitish epidermal trichomes (**Figs. 1-12**) that render the plant visible from far as grey patches in grasslands. Leaves are somewhat curled, glabrous above and tomentose beneath. Flowering has been recorded between November and August, but its peak is in April.

This species also appears to possess demulcent and emollient properties, and has been useful in the treatment of various diseases that attack human organs in the chest cavity. It has a pleasant smell and is much liked by Cape Coloured people who drink it as an infusion. The potential pharmacological interest of these folklore records led us to investigate the morphology, ultrastructure (**Figs. 1-12**) and chemical components of *P. serpyllifolia* aerial parts since no detailed study on these topics have not yet been reported.

The objective of this study was to analyze the essential oil of *P. serpyllifolia* extracted from leaves for commercial purposes and to test the remaining extract from distillation for antifungal and antibacterial activities.

MATERIALS AND METHODS

Plant collection

Plecostachys serpyllifolia (Berg) was collected from the Hogsback Forest about 20 km North of Alice, in the Eastern Cape Province, South Africa, at a height of approximately 1,200 m above sea level and transported to the laboratory in sealed plastic bags.

Oil extraction

Plant materials were collected from Hogsback and brought for distillation. One kilogram (kg) of leaf material was weighed and put in a 5-L flask and placed onto a heating mantle. Two litres of distilled water were poured in and the plant material was boiled for approximately 2 hours until no more oil was recovered. Steam generated from the water boiling in the flask, extracted the oil mixture from the plant material. The oil was collected in a graduated arm of a Clevenger apparatus and the oil samples were then subjected to GC-MS analysis. All experiments were done in triplicate.

Gas Chromatography-Mass Spectroscopy (GC-MS)

GC analysis was performed by inserting a wet needle of a sample material directly into the inlet (splitless mode) of a Hewlett Packard (HP) 6890 Gas Chromatograph. The injector temperature was 220ºC. Helium was used as carrier gas with a column (30 m x 0.25

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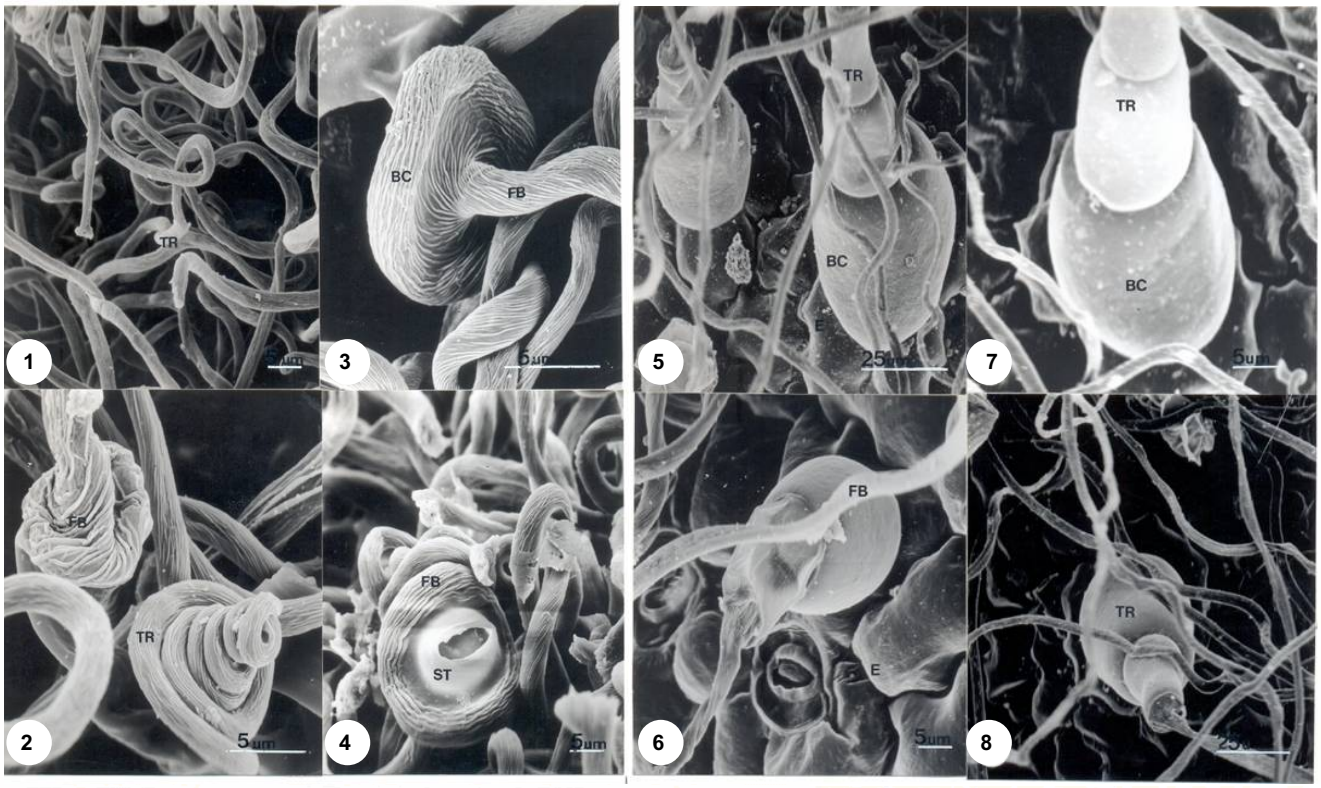
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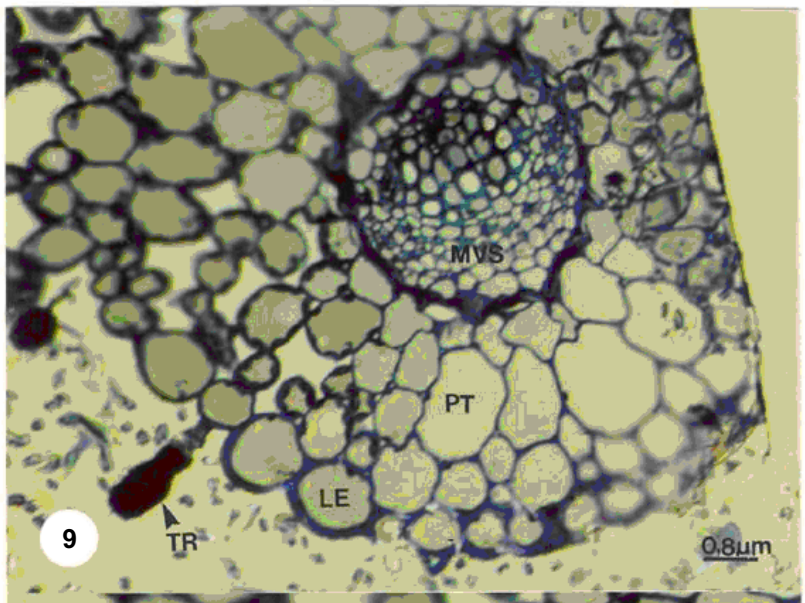
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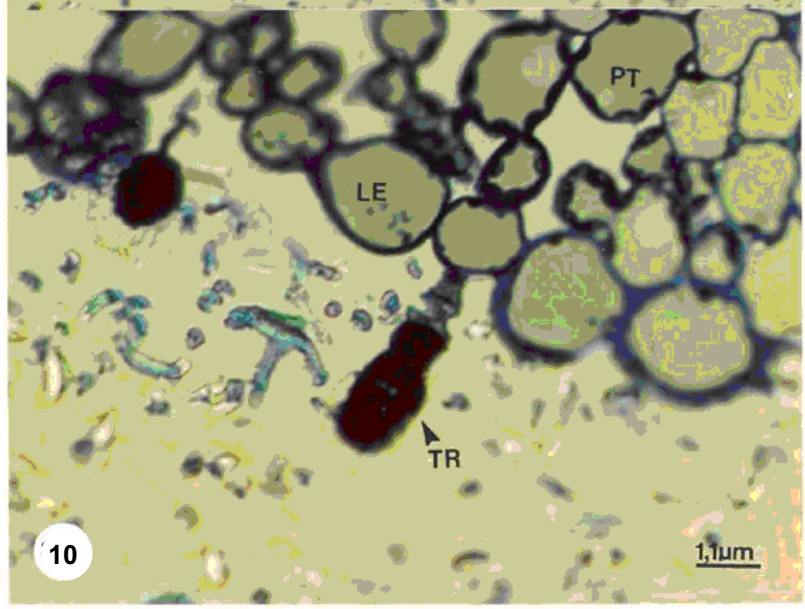
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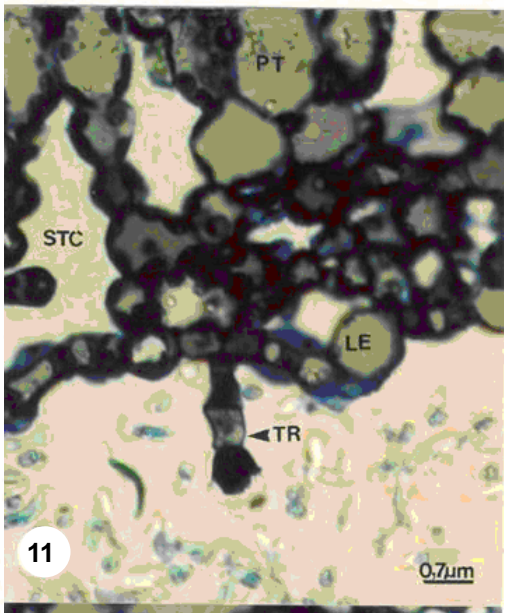
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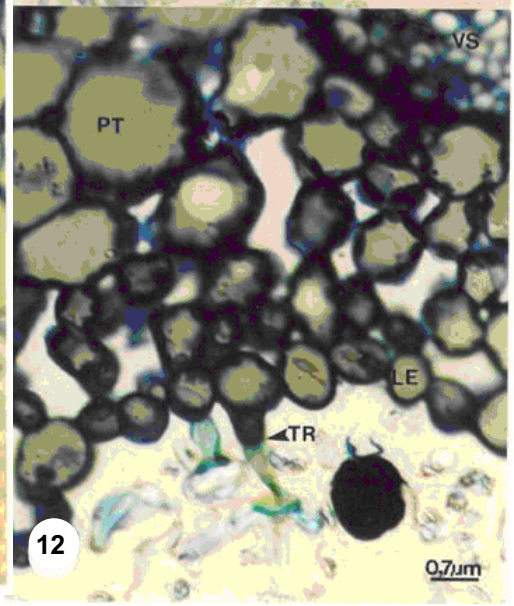
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Plate 1 (previous page) ***Plecostachys serpyllifolia*** **trichomes.** (**1**) Electron micrograph showing distribution of non-glandular trichomes completely obscuring the epidermis. (**2**) Electron micrograph of leaf epidermis showing high magnification of dehydrated coiled fibrous non-glandular trichome. (**3**) Electron micrograph of mature portion of a dehydrated fibrous trichome. (**4**) Electron micrograph of leaf displaying open stomata associated with fleshy, non-glandular trichome. (**5**) Electron micrograph of leaf showing the characteristic glandular trichome. (**6**) Electron micrograph of leaf showing a basipetal development of a trichome associated with the stomata. (**7**) Electron micrograph of leaf showing the basal cell and stalk cell of uniseriate trichome. (**8**) Electron micrograph of leaf showing basal portion of a trichome. (**9**) Transverse section of light micrograph of leaf section showing mature trichome with dark, amorphous material accumulating in the head of the trichome. The main vein is surrounded by a mestome sheath. (**10**) Higher magnification of transverse section of light micrograph of leaf section showing mature trichome with tanniniferous compounds accumulating in the head cells. (**11**) Transverse section of light micrograph of leaf section showing a three celled trichome with the apical cell filled with tanniniferous substances. (**12**) Higher magnification of a trichome showing dark material released by the trichome. Abbreviations: BC = cup-shaped basal cell, E = epidermis, FB = fibrous cell(s), LE = lower epidermis, MVS = main vascular system, PT = parenchymatous tissue, ST = stoma, STC = stomatal chamber, TR = trichome, VS = vascular system.

mm film thickness) was used. The oven temperature was programmed from 60ºC at 3ºC/min after a 3 min delay. A HP 5973 Series Mass Selective Detector (MSD) recorded Mass spectra. Essential oil constituents were identified on the basis of their retention times, and were co-injected with authentic compounds and analyzed by GC-MS.

Screening test for anti-microbial activity

Preparation of plant extracts

Aerial parts (stems and leaves) of *P. serpyllifolia* were collected and allowed to dry at 25ºC. *P. serpyllifolia* is an annual species, with the leaves drying off during the winter months. The leaves collected for experimentation are newly formed leaves approximately a month or two old. The leaves were cut into pieces of around a centimeter and then placed into conical flasks containning acetone, water or methanol. The plant materials were then extracted separately by shaking in acetone, water and methanol. Each extract was filtered using Whatman No. 1 filter paper and concentrated to dryness at reduced pressure with a rotary evaporator. The resultant residues were dissolved in their respective solvents to the required concentrations for minimum inhibitory concentration bioassay (Grierson and Afolayan 1999).

Preparation of agar-extract plates

Dilutions of each extract were poured into agar plates that were then streaked with bacteria or inoculated with an actively growing plug of fungi. Nutrient agar, NA (Oxoid) was used for bacteria and Potato Dextrose Agar, PDA (Biolab) for fungi were prepared by autoclaving and were allowed to cool to about 60ºC before the addition of the extracts. Agar medium containing the extracts at a final concentration of 0.1, 0.5, 1.0 or 5.0 mg/ml was poured into Petri dishes, swirled carefully until the agar began to set and left overnight for the solvents to evaporate. Plates containing agar medium with no extracts were used as controls. Each test tube was replicated three times.

Antibacterial testing

A total of ten selected bacterial species five Gram-positive, *Bacillus cereus*, *Bacillus subtilis*, *Micrococcus kristnae*, *Staphylococcus aureus* and *Stapylococcus epidermis* and five Gram-negative strains, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Serratia marcescens*, and *Shigella sonnei*, were obtained from collections of the Microbiology Department, Rhodes University. Each organism was maintained on NA slants (Biolab) and was recovered for testing by growth in nutrient broth (Oxoid) for 24 h at 37ºC. Before testing, each culture was diluted 1:100 with sterile nutrient broth.

The bacteria were streaked in radial patterns on the agar plates (Mitscher *et al*. 1972; Afolayan and Meyer 1997) and incubated at 30ºC for 24 h. The complete suppression of growth by a specific concentration on an extract was required for it to be declared active.

Antifungal testing

A total of four fungal species, *Rhizopus stolonifer*, *Synchephalastrum racemosum*, *Thamnidium elegans* and *Trichoderma koningii* were obtained from collections of the Microbiology Department, Rhodes University. Each culture was maintained on PDA and was recovered for testing by sub-culturing on fresh medium for 3 days. The prepared plates containing the extracts at the required concentrations were inoculated with plugs obtained from the actively growing margin of the fungal plates and incubated at 25ºC for 3 days. The diameter of fungal growth was measured and expressed as the means of percentage growth inhibition of three replicates (Barreto *et al*. 1997; Quiroga *et al*. 2001). Significant differences within the means of the treatments and the control were calculated using the LSD statistical test (Steel and Torrie 1960).

RESULTS

Characterization of essential oils extracted from *P. serpyllifolia* leaves

The essential oil was extracted during the vegetative growth on a monthly basis. There was not much difference in terms of the oil volume, but there was some difference in oil composition depending on the month. The essential oil obtained from *P. serpyllifolia* was pale yellow in colour and was characterized by a strong, spicy, resinous and slightly citrus-like odour. The spicy odour was due to the presence of the peppery-smelling monoterpenoid alcohol and terpinen-4-ol (**Table 1**).

The monoterpenes, and terpinolene contributed the pine-like taste while α-thujone and α- and β-terpinene supplied the citrus odour. The main oxidative agents of this plant were α-terpineol and terpinen-4-ol (**Table 1**).

These volatile compounds were identified using Gas Chromatography and were confirmed by Kovat's retention indices (Afolayan and Meyer 1997).

According to their retention time report, β-phellandrene appeared to be the dominant component followed by α-thujone and the least components were α-terpineol and α-terpinolene (**Table 1**).

Volatile leaf oil extracted from the leaves of flowering *P. serpyllifolia* consisted of different chemical constituents not found in the vegetative phase of the plant (**Table 2**), although there are some components that differ in their per-

Table 1 Essential oil composition of *Plecostachys serpyllifolia* (Berg).

Constituents	Retention time (min)	% of total
α-phellandrene	5.341	1.149
α-terpinene	5.560	6.865
Limonene	5.706	2.583
β-phellandrene	5.799	27.199
trans-β-ocimene	6.101	3.113
γ-terpinene	6.374	8.422
Terpinolene	7.012	2.366
α-terpinolene	7.192	0.942
β-thujone	7.422	9.500
(E)-4,8-dimethyl-1-3-7 nonatriene	7.568	1.379
α-thujone	7.670	22.591
propane dinitrile	8.333	1.044
terpinen-4-ol	9.083	4.296
α-terpineol	9.390	0.912
β-caryophyllene	15.140	1.996
Germacrene-D	16.651	2.240
Bicyclogermacrene	17.070	3.405

Table 2 Essential oil composition of leaves and stems of flowering *Plecostachys serpyllifolia* (Berg).

Constituents	Retention time (min)	% of total
α-pinene	4.11	13.60
2-β-pinene	4.80	25.63
myrcene	4.91	5.99
α-terpinene	5.42	1.04
limonene	5.65	3.92
γ-terpinene	6.23	1.68
α-thujone	7.27	1.06
β-thujone	7.51	2.45
terpinen-4-ol	8.95	2.89
α-terpineol	9.25	2.14
γ-elemene	12.91	4.28
β-caryophyllene	15.00	5.39
Germacrene-D	16.48	3.92
Bicyclogermacrene	16.87	6.03
(+) Spathulenol	18.74	2.20
Globulol	18.88	1.64
Viridiflorol	19.06	0.81

Components whose percentage < 0.81 are not included in the table above

centage and retention time. According to their retention time report, 2-β pinene appeared to be the dominant component followed by α-pinene and the least components were Viridiflorol and α-thujone (**Table 2**). The essential oils were identified by comparison of standards within the library of the GC-MS.

Antibacterial testing

The antibacterial assays of the aerial parts of *P. serpyllifolia* showed that the acetone extract inhibited the growth of all the Gram-positive bacteria tested at the minimum inhibitory concentration of 1 mg/ml. However, no inhibition of Gram-negative bacteria was observed (**Table 3**).

The methanol extracts effectively inhibited the growth of both Gram-positive and Gram-negative bacteria at the minimum concentration of 5 mg/ml with the exception of three Gram-negative bacteria, namely *Escherichia coli*, *Serratia marcescens* and *Shigella sonnei* (**Table 4**). The acetone and methanol extracts were very active against all the Gram positive bacteria tested.

The Gram-positive and Gram-negative bacteria seemed to be more resistant to the water extract with the exception of two Gram-positive bacteria, namely *Micrococcus Kristinae* and *Staphylococcus aurens*, which were inhibited at the minimum inhibitory concentration of 5 mg/ml (**Table 5**).

Table 3 Antibacterial activity of acetone extracts of *P. serpyllifolia* leaves.

Bacteria species	Gram +/–	MIC[a] (mg/ml)
Bacillus cereus	+	1.0
Bacillus subtilis	+	1.0
Micrococcus kristinae	+	1.0
Staphylococcus aurens	+	1.0
Staphyloccocus epidermidus	+	1.0
Escherichia coli	–	na
Proteus vulgaris	–	na
Pseudomonas aeruginosa	–	na
Serratia marcescens	–	na
Shigella sonnei	–	na

[a] Minimum Inhibitory Concentration
[na] Not active

Table 4 Antibacterial activity of methanol extract of *P. serpyllifolia* leaves.

Bacteria species	Gram +/–	MIC[a] (mg/ml)
Bacillus cereus	+	5.0
Bacillus subtilis	+	5.0
Micrococcus kristinae	+	5.0
Staphylococcus aurens	+	5.0
Staphyloccocus epidermidus	+	5.0
Escherichia coli	–	na
Proteus vulgaris	–	5.0
Pseudomonas aeruginosa	–	5.0
Serratia marcescens	–	na
Shigella sonnei	–	na

[a] Minimum Inhibitory Concentration

Antifungal testing

The extracts of *P. serpyllifolia* showed a range of antifungal activity against the fungi tested. Though not fungicidal, the water extracts from the shoots of *P. serpyllifolia* showed the least inhibitory activity against S*ynchephalastrum racemosum* and *Thamnidium elegans*, and apparently promoted the growth of *Rhizopus stolonifer* and *Trichoderma koningii* (**Table 6**).

In addition, methanol extracts showed 100% inhibition of *T. elegans* at the minimum concentration of 5 mg/ml. Although weakly fungicidal, the methanol extract showed appreciable growth inhibition of *T. koningii* and a high degree of inhibition against *S. racemosum* at a minimal concentration of 5 mg/ml (**Table 6**).

The acetone extract demonstrated significant antifungal activity against the four fungal species used. It was fungicidal (100% inhibition) on *Rhizopus stolonifer*, *T. elegans* and *T. koningii* at a minimal concentration of 5 mg/ml (**Table 6**). This extract showed a high degree of inhibition as the concentration increased from 0.1 to 5.0 mg/ml. Both the water and methanol extracts exhibited similar results against *R. stolonifer*.

R. stolonifer appeared to be more resistant to water and methanol extracts while an appreciable inhibition by the acetone extract was observed from the minimum inhibitory concentration of 0.5 to 5.0 mg/ml (**Table 6**)

The ability of the water extract to inhibit the growth of some Gram-positive bacteria and a number of fungal species is an indication of the possible broad-spectrum antimicrobial abilities of the plants. This could possibly ex-

Table 5 Antibacterial activity of the water extract of *P. serpyllifolia* leaves.

Bacteria species	Gram +/–	MIC[a] (mg/ml)
Bacillus cereus	+	na
Bacillus subtilis	+	na
Micrococcus kristinae	+	5
Staphylococcus aurens	+	5
Staphyloccocus epidermidus	+	na
Escherichia coli	–	na
Proteus vulgaris	–	na
Pseudomonas aeruginosa	–	na
Serratia marcescens	–	na
Shigella sonnei	–	na

[a] Minimum Inhibitory Concentration
[na] Not active

Table 6 Antifungal activity of extracts from *Plecostachys serpyllifolia.*

Extracts	Concentration (mg/ml)	Growth inhibition (%)			
		R. stolonifer	*S. racemosum*	*T. elegans*	*T. koningii*
Water	0.1	0	–21.2	7.2	0
	0.5	0	–12.0	–18.3	0
	1.0	0	–18.0	–7.10	0
	5.0	0	–9.2	–12.6	0
Methanol	0.1	0	–13.06	–11.17	0
	0.5	0	–3.80	6.20	0
	1.0	0	15.10	9.93	4.76
	5.0	0	53.69	100.0	48.73
Acetone	0.1	0	26.73	20.5	26.83
	0.5	20.63	58.81	49.44	61.90
	1.0	26.98	62.38	52.81	62.69
	5.0	100.0	78.22	100.0	100.0

Values represent the inhibition zone (mm) and are an average of three triplicates.
R. stolonifer - Rhizopus stolonifer; *S. racemosum - Synchephalastrum racemosum*; *T. elegans - Thamnidium elegans*; *T. koningii - Trichoderma koningii*

plain the use of plants to treat a number of diseases.

DISCUSSION

The essential oil from *P. serpyllifolia* were characterized by the presence of monoterpenes namely limonene (2.58%), terpinolene (2.36%), γ-terpinene (8.42%) and others. Secondary metabolites extracted from *P. serpyllifolia* leaves examined by GC/MS revealed that the dominant components were β-phellandrene (27.19%), α-thujone (22.59%), β-thujone (9.50%) and γ-terpinene (8.42%) while those in flowering *P. serpyllifolia* were β-pinene (25.63%), α-pinene (13.60%), bicyclogermacrene (6.03%) and myrcene (5.99%). The essential oil composition of our material was quite different from that already reported by Tucker and Maciarello (1996), who showed that the dominant components were β-caryophyllene (21.29 ± 10.90%), β-pinene (14.99 ± 8.40%) and sabinene (10.73 ± 4.98%).

It is well known that these essential oils can also be used for medicinal purposes as there is an increasing trend worldwide to integrate traditional medicine with primary health care. *Bacillus cereus* has been implicated in food poisoning (Granum and Lund 1997), *B. subtilis* has been associated with a range of clinical conditions, food spoilage and incidents of food-borne diseases such as gastroenteritis (Salkinoja-Salonen *et al*. 1999). *Micrococcus Kristinae* is known to cause abscesses. *Escherichia coli*, *Staphyllococcus aurens* and *Pseudomonas aeruginosa* cause diseases like mastitis, abortions and upper respiratory complications (Fraser 1986). *Serratia marcescens* is another important nasocomial pathogen, which is often resistant to multiple antimicrobial agents (Yum *et al*. 2002). Similarly, the fungi used in our study are of human and veterinary importance.

Evaluation of the antibacterial properties of *P. serpyllifolia* using acetone extract essential oils indicates a significant activity against all the Gram-positive bacteria used with a minimum inhibitory concentration (MIC) as low as 0.1 mg/ml. Although the extracts were generally less active against the Gram-negative bacteria, *Proteus vulgaris* and *Pseudomonas aeruginosa* were the most susceptible bacteria inhibited by methanol extract at 5 mg/ml. *Escherichia coli*, *Serratia marcescens* and *Shigella sonnei* were the most insensitive strains of all the bacteria used in this study. In fact, Gram-negative bacteria, in particular *E. coli*, are frequently reported to develop multi drug resistance to many of the antibiotics currently available in the market (Alonso *et al*. 2000; Sader *et al*. 2002).The observed lower degree of activity of the extracts against the Gram-negative bacteria is not surprising since, in general, these bacteria are more resistant than the Gram-positive ones (Rabe and van Staden 1997; Grierson and Afolayan 1999). The probable difference between the acetone and water extracts could be attributed to the polarity of the medium in which the extractions were performed. Water tends to be more polar and extracts would be limited if the extracts where not boiled. The boiling process employed by traditional healers tends to break the cellulose cell walls which could increase the product output. Acetone on the other hand, being less polar than water, has a tendency to damage, break or weaken the cellulose cell walls of the oil glands, in so aiding or assisting the extraction process.

The results showed a lower antibacterial activity when water extract was added to the Agar. Plant extracts are traditionally prepared with water, and it is unlikely that the traditional healer adequately extracts those compounds that are responsible for activity in the acetone and methanol extracts. This could also be due to that fact that the traditional healers are resource-limited and in addition, methanol is not readily for use by lay people.

The extracts of *P. serpyllifolia* showed a range of antifungal activity against the fungi tested. The acetone extract was the most active with inhibitory activity ranging from 20.5% against *Thamnidium elegans* to 100% against *Rhizopus stolonifer*, *Thamnidium elegans* and *Trichordema koningii* at 5 mg/ml, which was the highest concentration tested. Eloff (1998) also observed in his investigations that acetone extract gives the best results in terms of quantity and diversity of compounds, and the number of inhibitors extracted.

The methanol extract also exhibited 100% inhibition against *T. elegans* but *R. stolonifer* was found to be virtually insensitive to methanol and water extracts. The water extract showed the least antifungal activity with no significant effect on the growth of the organisms.

The activity of plant essential oils in water extracts against Gram-negative bacteria is noteworthy. This is because; most workers (Martin *et al*. 1993) have generally reported that water extracts of plants do not have much activity against bacteria.

CONCLUSION

P. serpyllifolia possesses antibacterial and antifungal properties. These observations justify the homogenization process (grinding) in water employed by the traditional healers during the preparation of their medicine for the treatment of microbial infections. The ability of the extracts to inhibit the growth of all Gram-positive and a number of Gram-negative bacteria used, as well as the growth of fungal species, is an indication of the potential of the herbal medicine as a source of broad spectrum antimicrobial agent. This probably explains the use of the herb by traditional herbalists of the Eastern Cape in South Africa, against a number of infections. At present, South Africa is rich in biodiversity, but relative to most first-world countries it is poor in biotechnology. It is important that the latter be strengthened to make full use of the potential of all our resources.

This type of information is necessary in the current search for novel antimicrobial drugs. Many species with medicinal value have been irretrievably lost, or are on the verge of extinction because of ruthless digging and careless collection. Looking into the future supply of raw materials, conservation of natural vegetation, protection of species containing compounds invaluable to the well-being of mankind, and cultivation of useful medicinal species should be put into practice without delay. The essential oils as well as their respective antimicrobial activities, obtained from the *P. serpyllifolia* leaves indicate a suitable source of alternative medicines as compared to contemporary medicines. The sourcing of these essential oils provide us with a broader knowledge of the availability of these oils. Currently the entire world is placing major emphasis on Herbal or traditional medicines. This work would aid if future need of the isolated essential oils become a necessity.

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